Laser Desorption Substrate Effects

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Information about energy transfer during laser desorption from surfaces is important for the theoretical description of the desorption mechanism and necessary for optimizing applications of laser desorption mass spectrometry. We present a simple method based on laser multiphoton ionization to estimate the internal energy of a laser-desorbed neutral biomolecule, the amino acid tryptophan. The internal energy of neutral laser-desorbed molecules affects ion fragmentation as well as the minimum ionization energy necessary for detecting tryptophan photoions. Data on tryptophan desorbed from glass, graphite, stainless steel, as well as from a silylized glass substrate, were recorded. The silylation of glass reduced the internal energy of desorbed tryptophan dramatically. (J Am Soc Mass Spectrom 1997, 8, 525–531) © 1997 American Society for Mass Spectrometry

The internal energy of laser-desorbed or sputtered molecules is a much sought after quantity, especially in the context of mass spectrometric analysis of complex and labile biomolecules, where gas-phase neutral and ionic species are only accessible by desorption or sputtering methods. Many different methods for volatilizing and ionizing biomolecules have been developed [1–11]. All of these methods aim to produce gas-phase molecules with minimal decomposition, that is, with the least possible amount of internal energy and the highest possible fraction of parent ions in the mass spectrum.

Mass spectrometric methods that use laser postionization [4–8] allow the greatest control over the experimental parameters affecting fragmentation, as a result of temporally and spatially separated desorption and ionization events. However, there is still a trade-off between absolute signal and the absence of fragmentation: a high ionization laser irradiance produces intense signals but with significant fragmentation. A reduced irradiance often yields mass spectra dominated by molecular ions, but they are much less intense.

One of the drawbacks of laser postionization is that the mass range appears to be limited, for example, to < 2000 u in the case of peptides [12, 13]. The reasons for this limitation are still not entirely clear. Schlag et al. [14] proposed that larger molecules fail to be ionized because of the recombination of an initially created charge pair and thermalization of the excess energy. Others have challenged this view. Anex et al. [15] demonstrated that it is possible to generate parent ions of functionalized perfluorinated polyethers extending to 7000 u by using laser desorption in conjunction with jet cooling, followed by two-photon ionization of a terminal chromophore. Becker and Wu [16] hypothesized that the upper mass limit is a result of fragmentation due to a high internal energy acquired in the desorption event; these authors also pointed out that Schlag's group itself had observed masses above 15,000 u in a supersonic jet laser postionization experiment.

Supersonic jet entrainment of desorbed molecules is indeed the most successful approach for avoiding fragmentation, in particular if a low ionization laser irradiance is used (Table 1) [17–19]. Matrix-assisted laser desorption (MALD) was also reported to produce neutral molecules with low internal energy [16, 20, 21]. This has been attributed to cooling of the internal degrees of freedom via collisions with matrix species in the desorption plume. Reilly and Reilly [22] used rhodamine B and glycerol mixtures as a matrix to promote the desorption of tryptophan. Their laser ionization mass spectra showed a decrease in fragmentation with increasing desorption pulse energy. Mowry and Johnston [23] measured the internal energy of alkylamines laser-desorbed from various matrices.

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They found internal energies between 0.6 and 0.9 eV, corresponding to temperatures close to the sublimation temperatures of the matrices used. These measurements were done by observing metastable fragmentation of several alkylamines formed after single photon ionization at 9.67 eV, comparing laser desorption data with calibration measurements obtained from hot vapors. Such a calibration is unfortunately not possible for nonvolatile or thermally labile biomolecules. Desorption of tryptophan from a cryogenic ice matrix was studied by Elokhin et al. [24]. These authors found that the cooling did not approach the levels achieved in supersonic molecular beams. This was evident from the residual fragmentation in the mass spectrum and from unresolved features in the optical spectrum. In general, collisional cooling by co-desorbing matrix in MALD is expected to be rather inefficient due to an unfavorable C_p/C_v ratio [25]. The effects of both laser desorption and thermal desorption on molecular fragmentation were studied by Ayre et al. [26]. Postionization time-of-flight mass spectra obtained by using laser-induced desorption of tryptophan deposited on indium foil were compared to data using thermal desorption of tryptophan from tantalum foil. Thermal desorption was found to be a much softer method. A summary of the mass spectrometric fragmentation of the amino acid tryptophan at different desorption and postionization conditions is given in Table 1.

The goals of the present study were twofold. First, we wanted to use photoionization mass spectrometry to determine the internal energy of a nonvolatile biomolecule. We found that the amino acid tryptophan

Table 1. Degree of tryptophan fragmentation for different experimental conditions

Desorption method	Desorption energy	Substrate	Sample layers (ML)[a]	Ionization wavelength	Ionization irradiance (W/cm^2)	Jet cooling (yes/no)	M/F[b]	Reference
10.6 μm	N/A	PE/Pt matrix [c]	N/A	260 nm	2.62×10^{12} (ns pulses)	Yes	~ 0.6	Aicher et al. [28]
					5.24×10^{11} (fs pulses)		~ 0.15	
10.6 μm	N/A	PE/Ag matrix[c]	N/A	270 nm	2.63×10^3	Yes	7.08	Dey et al. [27]
7-keV Ar^{+}[d]	6×10^{12} ($ions/cm^2$)	Indium foil	N/A	118 nm	N/A	No	0	Ayre et al. [26]
				266 nm	4×10^8		0	
				335 nm	6×10^{10}		0	
Thermal (150 °C)	N/A	Tantalum foil		118 nm	N/A		0.57	
				266 nm	4×10^8		0.23	
532 nm	0.05 mJ	Rhodamine B/ glycerol	N/A	266 nm	N/A	No	0	Reilly and Reilly [22]
							~ 0.61	
	2.00 mJ	matrix					~ 0.75	
	0.05 mJ	Rhodamine B/ glycerol	N/A	286.75 nm				
	2.00 mJ	matrix					~ 2.50	
248 nm	10^5–10^8 W/cm^2	N/A	Multi-layers	290.5 nm	3.5×10^7	No	~ 0.3	Spengler et al. [42]
Thermal (230 °C)	N/A	Brass	N/A	290 nm	N/A	Yes	7.26	Rizzo et al. [34]
1064 nm	3.3×10^4 (W/cm^2)	Aqueous cryogenic matrix	N/A	286 nm	2×10^4	No	7.51	Elokhin et al. [24]
				70 eV[e]	N/A		0.06	
10.6 μm	6×10^6 (W/cm^2)	Glass	10–20	290 nm	1.2×10^6	No	0.16	This work
		Stainless					0.50	
		Graphite					1.16	
		Silylized glass					1.68	

[a] ML = monolayers; N/A = not available.
[b] M/F = ratio of parent ion (m/z 204) intensity to fragment (m/z 130) intensity.
[c] PE = polyethylene.
[d] Desorption by argon ion bombardment rather than with laser light.
[e] Ionization by 70-eV electron impact rather than photoionization.

fulfills all requirements to serve as a probe molecule for such a measurement. Second, we wanted to study the influence of various substrates on the internal energy of laser-desorbed tryptophan, considered by many to be a model for larger biomolecules such as peptides or proteins [22, 24, 26–28].

Experimental

Materials

Several different materials were employed as substrates. Glass, stainless steel, and graphite (Goodfellow Cambridge Limited, England) were machined into disks with ≈ 12 mm diameter and 3 mm thickness. Silylation was carried out by immersing clean glass in octadecyltrichlorosilane protected from humidity by N_2 purging overnight. The silylized glass was then cleaned with organic solvents (dichloromethane, acetone, and methanol). L-tryptophan and octadecyltrichlorosilane were obtained from Fluka (Buchs, Switzerland). Tryptophan is oxidized slowly in aqueous solution over the course of days [29], and fresh solutions were therefore prepared weekly.

Sample Preparation

All materials were used as received. Tryptophan was dissolved in deionized water as a dilute solution ($\approx 5–10 \times 10^{-10}$ M). For sample deposition, the substrates were fixed on an aluminum sheet with double stick tape. A fine spray of the tryptophan solution was directed at the substrates by using an air brush. At the same time, a heat gun was directed at the substrate, so that the substrate surface was always dry during the sample preparation process. This procedure produced a homogeneous thin sample layer. The sample thickness was estimated to be 10–20 monolayers (ML) by measuring the amount of solution sprayed on the target, and by comparing the UV absorption of the film with that of dilute tryptophan solutions. Because it is the substrate surface that is heated by pulsed IR laser radiation, desorption of multilayers will still be governed by the interaction between the substrate and analyte. Some collisions will occur in the desorption plume after complete desorption of 10–20 ML, but they only weakly influence the velocity distributions and have little or no effect on the internal energy [25]. Repeat measurements on replicate samples by using identical experimental conditions showed virtually identical mass spectra.

Laser Mass Spectrometer

For mass spectrometric analysis, a home-built two-step laser mass spectrometry (L2MS) apparatus was used [30]. The output of the CO_2 laser used for desorption (Alltech model 853 MS, Lübeck, Germany) was directed into the system by a set of mirrors and a ZnSe lens. The irradiance was adjusted to $\sim 6 \times 10^6$ W/cm^2 by two irises and by the size of the laser focus. This laser (10.6-μm wavelength, ≤ 100-ns pulse width) was operated at a 4-Hz repetition rate to avoid cumulative heating of the sample. Because the infrared laser pulses desorbed the tryptophan layer almost completely, a fresh sample area was exposed to the CO_2 laser beam for each desorption shot by rotating the sample. The ionizing laser radiation was produced from an optical parametric oscillator laser (MOPO-730D20, Spectra-Physics Lasers, Inc., Mountain View, CA), pumped by the third harmonic of a pulsed Nd:YAG laser (model GCR 230). The wavelength can be varied nearly continuously from 220 to > 2000 nm. In this work, the wavelength range used was between 266 and 335 nm, corresponding to photon energies from 4.66 to 3.70 eV. The UV laser light passed the plume of desorbing molecules 3.5 mm above the sample surface and was delayed with respect to the CO_2 laser pulse by 20–40 μs. The UV power was adjusted by a motor controlled polarizer (MGTYB15, Karl Lambrecht, Chicago, IL). The UV laser irradiance was always kept at 1.2×10^6 W/cm^2. Inspection of Table 1 shows that some fragmentation will always occur at this irradiance, even for jet-cooled tryptophan. This was important in our study for observing molecular ion to fragment ion signal ratios. Mass spectrometric analysis was achieved by using a reflectron time-of-flight instrument (R. M. Jordan Co., Grass Valley, CA).

Mass Spectrometric and Electronic State Properties of Tryptophan

Tryptophan was employed as a probe molecule in this study because its mass spectrometric fragmentation pathways are well established [27, 28, 31]. Because this amino acid dominates the near ultraviolet absorption and fluorescence of many proteins, the electronic spectroscopy and photophysics of tryptophan and its derivatives have also been intensely studied [32–36].

For correlating mass spectrometric fragmentation of photoions with internal energy, it is important to know whether fragments are formed in the ionic state only or whether there are contributions from dissociation products originating from photoexcited neutrals or from pyrolysis products created during the desorption [37]. Comparisons between femtosecond and nanosecond UV laser ionization [28] show that tryptophan fragments are not produced from excited neutrals but rather from further excitation of the molecular ion by the so-called ladder-switching mechanism [17, 27]. The appearance of fragment ions depends on the total energy, that is, on the number of photons that the molecule has absorbed. For example, the peak at m/z 130, which was always seen in our spectra, is the result of a three-photon process at wavelengths greater than 270 nm (4.6 eV) [27], which corresponds to an appearance energy between 9.2 and 13.8 eV.

To rule out the possibility of pyrolytic fragmentation, a temperature-programmed desorption (TPD) experiment with tryptophan dosed on a quartz surface was carried out in a separate ultra-high vacuum apparatus, by using a heating rate of 0.3 K/s. We found that a coverage of 10–20 ML of tryptophan on silica desorbed intact between 400 and 500 K, with only a very small contribution from products that could either be attributed to thermal decomposition on the substrate or to other dissociative processes occurring on filaments, chamber walls, and so forth. A minor conclusion from these TPD data is that tryptophan, which is considered a thermally labile and nonvolatile amino acid, can be sublimated under high vacuum conditions, even at low heating rates. Perhaps the assumption that tryptophan is thermally labile is related to the fact that melting and boiling points are commonly determined under atmospheric conditions where oxidative decomposition can occur.

Figure 1 shows the energy diagram of tryptophan. Photoionization of tryptophan occurs by 1 + 1 resonance enhanced multiphoton ionization (REMPI) [27], where the neutral molecule is excited by a first photon and ionized (IP = 7.2 eV [32]) by a second photon. The frequency of the 0–0 transition of tryptophan is 34873 cm^{-1} (4.32 eV) [34]. The indole chromophore possesses two low lying singlet states, designated as L_a and L_b. Their energy difference was measured to be 35.9 cm^{-1} [34]. In this work tryptophan was not jet cooled, and the exact wavelength was not expected to be decisive for the ionization efficiency. A fine scan of 45 cm^{-1} around the threshold energy for the appearance of photoions confirmed that the L2MS spectra were not influenced by these close lying states.

If the tryptophan molecule is in the electronic and vibrational ground state, the photon energy required for exciting the 0–0 transition is 4.32 eV. If the molecule is vibrationally hot, the internal energy will contribute to the excitation of the intermediate electronic state. We recorded wavelength resolved mass spectra (UV/MS) over a wide range of photon energies to estimate the tryptophan internal energy.

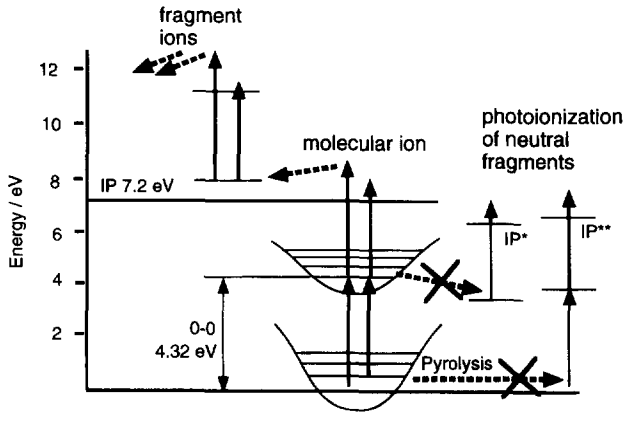

Figure 1. Energy diagram of tryptophan and its possible fragmentation pathways.

Results and Discussion

Figure 2 shows the UV/MS spectra for tryptophan laser-desorbed from untreated glass using a photon energy range from 3.70 (335 nm) to 4.66 eV (266 nm). The peak at m/z 204 is the molecular ion, but all spectra are normalized to m/z 130, which is the dominant peak at single photon energies above 4 eV. The signal at m/z 130 [M–CH(NH_2)COOH] is the result of breaking the C_α–C_β bond and was always observed at the UV irradiance used, consistent with all other photoionization work on tryptophan (see Table 1). Even with molecular beam cooling of neutral tryptophan, this fragment is not completely suppressed [34, 38]. The peak at m/z 131 can be assigned to a McLafferty-type rearrangement product or to methyl indole, perhaps created during sample preparation [34]. Peaks at m/z 220 and 236, 16 and 32 u above m/z 204, were also observed in Figure 2, especially at low ionization energies. They are interpreted as hydroxytryptophan and dihydroxytryptophan, probably produced by hydroxylation in aqueous solution [29]. Experimentally, it was found that the intensity of the 220-u peak grew by a factor of ≈ 2 relative to the 204-u signal upon exposure of tryptophan solutions to air for several days. By analogy to the difference in UV absorption between benzene and phenol [39], the maximum UV absorption of hydroxytryptophan is expected to lie at a longer wavelength. As a result, relatively high signals of hydroxytryptophan were obtained close to the threshold energy for appearance of tryptophan photoions.

Although the two-photon energy in all spectra shown in Figure 2 was large enough to overcome the ionization potential, single photon energies below 4.32 eV are smaller than that required to excite the 0–0 transition in tryptophan. The fact that intense signals were observed down to 3.7-eV photon energy clearly indicates that internal energy contributes to the excitation of the L_a/L_b state. At one-photon energies below 3.7 eV, the signal intensity dropped dramatically, which

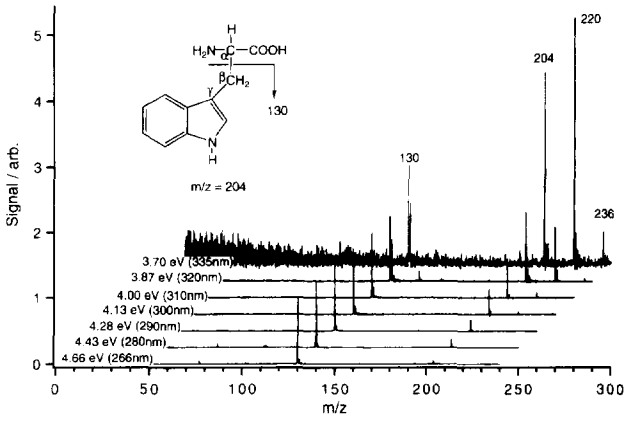

Figure 2. Wavelength resolved L2MS spectra of tryptophan desorbed from untreated glass substrates. The spectra are normalized to the fragment signal at m/z 130.

is evident from the much larger noise in the normalized data. To reach the first excited state of tryptophan with this UV photon energy, the tail of the internal energy distribution must exceed 0.62 eV. The fraction of internally hot tryptophan is probably underestimated from the data because the Franck–Condon overlap from a vibrationally excited ground state to the vibrationless L_a/L_b state is expected to be poor. Our finding supports the hypothesis of Becker and Wu [16] that the internal energy of laser-desorbed biomolecules can be quite substantial, which can contribute significantly to fragmentation.

The measured ratio of the parent ion intensity to the m/z 130 fragment ion intensity (M/F) in Figure 2 increases from 0.04 at 4.66 eV to $M/F = 3.2$ at 3.7 eV. Although this may reflect a decreasing cross section for absorption of a third photon by the molecular ion, we interpret this result to be the consequence of a decreasing total energy deposition. If this interpretation is correct, less fragmentation should be seen for species desorbed with less internal energy, if all other experimental conditions are identical. The M/F value depends particularly strongly on the ionization laser irradiance (see Table 1). For example, Grotemeyer and Schlag [17] obtained a reduction of fragmentation by a factor of 5 when the UV irradiance was reduced from 5×10^6 W/cm^2 to 5×10^5 W/cm^2.

UV/MS spectra were therefore recorded using silylized glass as a substrate; the results are shown in Figure 3. The UV photon energy was between 4.00 (310 nm) and 4.43 eV (280 nm). Again, the molecular ion at m/z 204 as well as the fragment at m/z 130 were observed. At an ionization laser irradiance of 1.2×10^6 W/cm^2, identical to the one used for the data presented in Figure 2, the M/F ratio for silylized glass ranged from 0.47 at 4.43 eV to 1.8 at 4.13 eV, to almost infinity at 4.0 eV. At all energies, the measured M/F ratio for silylized glass was substantially larger than that of untreated glass. At 4.13 eV (300 nm) the signal-to-noise ratio started to become very poor and the tryptophan signal was close to the detection limit. At

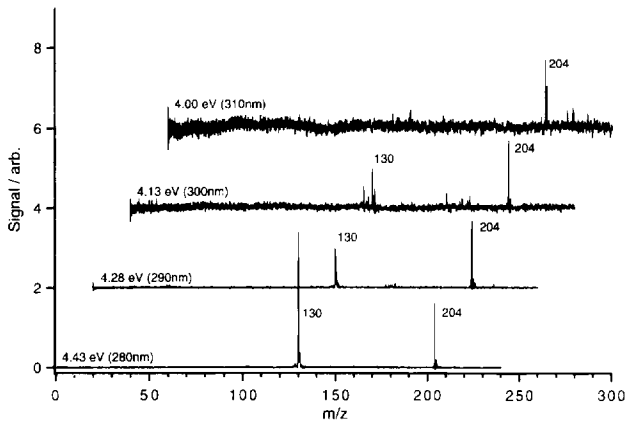

Figure 3. Wavelength resolved L2MS spectra of tryptophan desorbed from silylized glass substrates. The spectra are normalized to the molecular ion signal at m/z 204.

this photon energy, a contribution of ~ 0.19 eV of internal energy is required for reaching the first excited state. We interpret these findings to be due to a lower internal energy of tryptophan desorbed from silylized glass compared to desorption from untreated glass. Assuming thermal energy distributions [40, 41], the reduction in internal energy is about threefold.

It is reasonable to assume that the termination of a surface has an important influence on the energy flow from the substrate to the adsorbate. We believe that hydrogen bonds between adsorbed tryptophan and silanol groups at the glass surface represent favorable channels for energy flow [40]. Upon silylation of the glass surface, the surface hydroxyl groups react and will no longer be available for hydrogen bond formation, effectively suppressing this channel. The desorption/photoionization of perfluorinated polyethers described by Anex et al. [15] is similar in the sense that no polar interactions such as H bonds were possible between the polyethers and the substrate, a piece of charcoal. These compounds could be detected up to high molecular weight, which is consistent with a reduced internal energy content of desorbed neutrals. An alternative explanation is that molecules simply desorb at lower temperatures from apolar surfaces.

We also performed velocity distribution measurements by probing laser-desorbed neutral tryptophan molecules at different delay times of the ionization laser pulse, at a fixed distance (3.5 mm) between the desorption spot on the surface and the ionization volume, in the direction normal to the substrate surface. The data are displayed in Figure 4 as time-of-flight distributions of the integrated parent ion signal. The UV wavelength used was 4.28 eV (290 nm). Velocity distributions of tryptophan desorbed by UV laser radiation were previously studied by Spengler et al. [42]. Assuming a cosine angular distribution and a Maxwellian velocity distribution they found translational temperatures of about 300 K at a desorption wavelength of 248 nm; at 193 nm, the fitted translational temperature was about 1000 K. This behavior probably results from absorption of the UV by the tryptophan itself, resulting in a desorption process mediated by electronic excitation, as opposed to infrared laser-induced thermal desorption used in this work.

Our data are summarized in Figure 4. The upper panel shows velocity distributions using untreated glass (solid circles) and silylized glass substrates (open circles); the lower curve is from a graphite substrate (squares), another apolar surface. The translational energy of desorbed tryptophan was quite low for all substrates. One possible explanation for this is that the molecules leave the surface only some time after the CO_2 laser pulse. However, experiments employing different flight distances showed that this was not the case. A plot of the maximum velocity versus flight distance (not shown) indicated that there was no induction time. The most probable velocity for trypto-

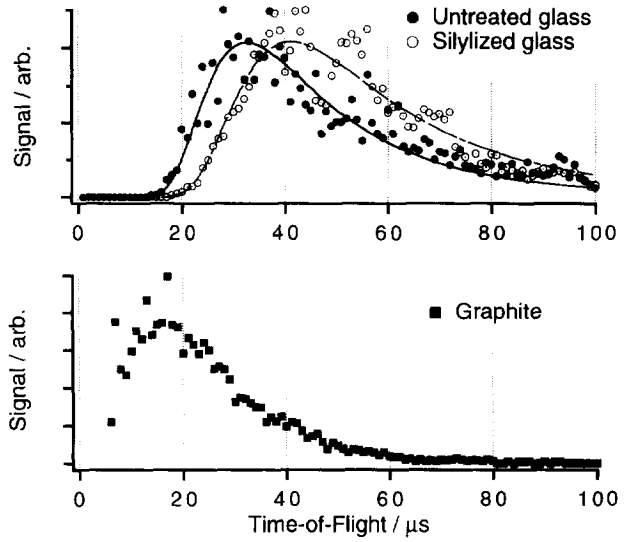

Figure 4. Time-of-flight distributions of laser-desorbed neutral tryptophan, for a flight distance of 3.5 mm. The tryptophan film thickness was 10–20 monolayers. Points are 40 shot averages. (**a**) Desorbed from treated (open circles) and untreated glass surfaces (solid circles). The lines are fits to Maxwell–Boltzmann velocity distributions, with fitted translational temperatures of ≈ 70 K (glass) and ≈ 40 K (silylized glass). (**b**) Desorbed from a graphite surface (squares).

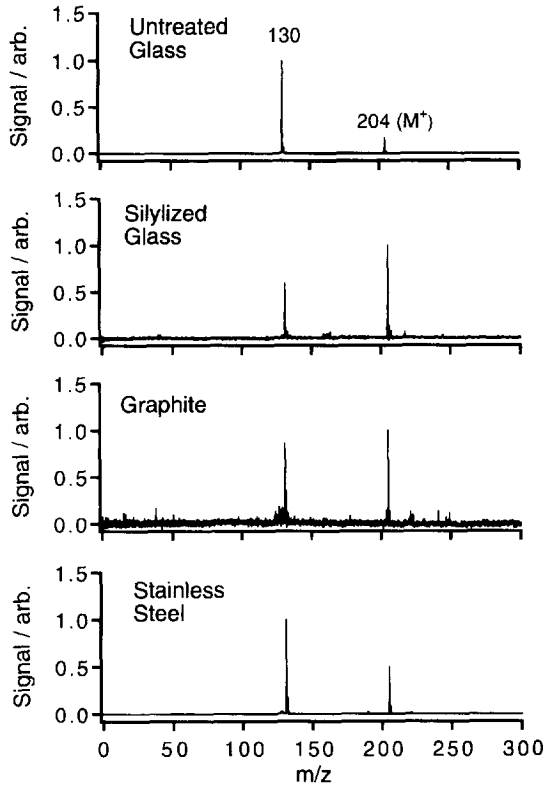

Figure 5. The L2MS spectra of tryptophan ionized by 4.28-eV (290-nm) photons by using different substrates: untreated glass, silylized glass, graphite, and stainless steel.

phan desorbed from glass was ≈ 100 m/s. The velocity distribution of tryptophan desorbed from silylized glass was broader, with a lower maximum velocity (≈ 80 m/s) than that of untreated glass. Although the experimental error was about ±18%, mainly due to the uncertainty of the flight distance, the difference was significant and was consistently found in repeat measurements. This behavior mirrors our findings for the internal energy, which was also higher in the case of untreated glass. The data for glass and silylized glass appeared to be thermal and could be fitted to Maxwell–Boltzmann velocity distributions (lines). Tryptophan desorbed from graphite showed a non-thermal behavior with a most probable desorption velocity of 220 m/s. The reason for this is unknown, but a different heating rate or a different desorption mechanism may be responsible. For example, graphite itself may desorb following CO_2 laser irradiation.

Figure 5 compares L2MS spectra of tryptophan desorbed from four different substrates: untreated glass, treated glass, graphite, and stainless steel. Stainless steel was included because it is often used as a substrate for laser desorption mass spectrometry. The tryptophan layer thickness was ~ 10–20 ML in all cases, the ionization laser irradiance was 1.2×10^6 W/cm^2, and the photon energy was 4.28 eV (290 nm), 0.04 eV below the 0–0 transition. Not much internal energy is required for efficient 1 + 1 REMPI to occur in this case, which is evident from a good signal-to-noise ratio for the data shown in Figure 5. At 290 nm, the fragment at m/z 130 must result from a three-photon process [27].

The M/F ratios for the polar substrates, untreated glass, and stainless steel were relatively low: M/F = 0.16 and 0.51, respectively. In contrast, a high M/F was obtained by using silylized glass (M/F = 1.7) and graphite (M/F = 1.5), both apolar surfaces. The change between untreated and silylized glass is striking: the M/F ratio increases by 1 order of magnitude after this simple surface treatment.

Conclusions

Mass spectrometric fragmentation following multiphoton ionization is greatly affected by the internal energy of the analyte. For analytes that need to be volatilized by desorption or sputtering methods, the internal energy can be high and may in some cases prevent the observation of intact parent ions. Many different strategies exist to minimize fragmentation; low ionization laser irradiance and collisional cooling by using supersonic jet entrainment are the most effective. In the absence of collisional cooling, the internal energy is determined by the substrate or matrix from which desorption takes place. We show that the internal energy content of laser-desorbed tryptophan can be estimated by using wavelength resolved L2MS spectra. We also demonstrate that its internal energy can vary by a factor of 3 depending on the substrate and that by proper choice of the substrate fragmentation can be reduced by an order of magnitude. Of the substrates studied, silylized glass substrates led to the smallest

amount of fragments in the mass spectra. We believe that elimination of surface sites for hydrogen bonds with polar adsorbates leads to a dramatic reduction in internal energy transferred to the adsorbate during laser desorption.

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